SPECIFICITY OF TARGETING IN RNAi AND miRNA PATHWAYS

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ABSTRACT

This review systematically covers sequence-specific gene regulation by miRNAs and dsRNA-derived siRNAs in animals and plants from the perspective of target RNA recognition, potential for non-target (off-target) effects, and reliable determination of biological effects of small RNAs in animals and plants. I will review sequence complementarity between target RNA and small RNA (siRNA or miRNA), including tolerance to mismatches, parameters influencing sequence complementarity (and target recognition and repression) and discuss specificity of targeting by miRNAs and off-targeting by siRNAs. In addition, I will discuss reliable identification of target RNAs (and, eventually, biological effects). Accordingly, the text is divided into the following four sections: (I) Small RNA:target RNA base pairing, (II) Other key factors influencing target recognition and repression, (III) Off-targeting – causes and remedies, (III) Smal RNA target identification.

Introduction

Within the complex world of RNA silencing, two related yet distinct pathways exist in animals and plants: RNA interference (RNAi) and microRNA (miRNA) pathways (Fig. 1). Both pathways employ small RNAs loaded on Argonaute proteins as sequence-specific guides for post-transcriptional repression. The elementary difference between these two pathways is that miRNA pathways employ genome-encoded small RNAs with defined sequences (i.e. miRNAs can be annotated) while RNAi is initiated by processing long double-stranded RNA (dsRNA) into a mixture of short interfering RNAs (siRNAs). Thus, the miRNA pathway in a cell employs a population of miRNA molecules that can be clustered based on unique sequences, corresponding to specific positions in miRNA precursors. In other words, the major distinction between RNAi and miRNA pathways is the origin of small RNAs and their information content. In terms of their mode of action, siRNAs and miRNAs can be in some cases indistinguishable.

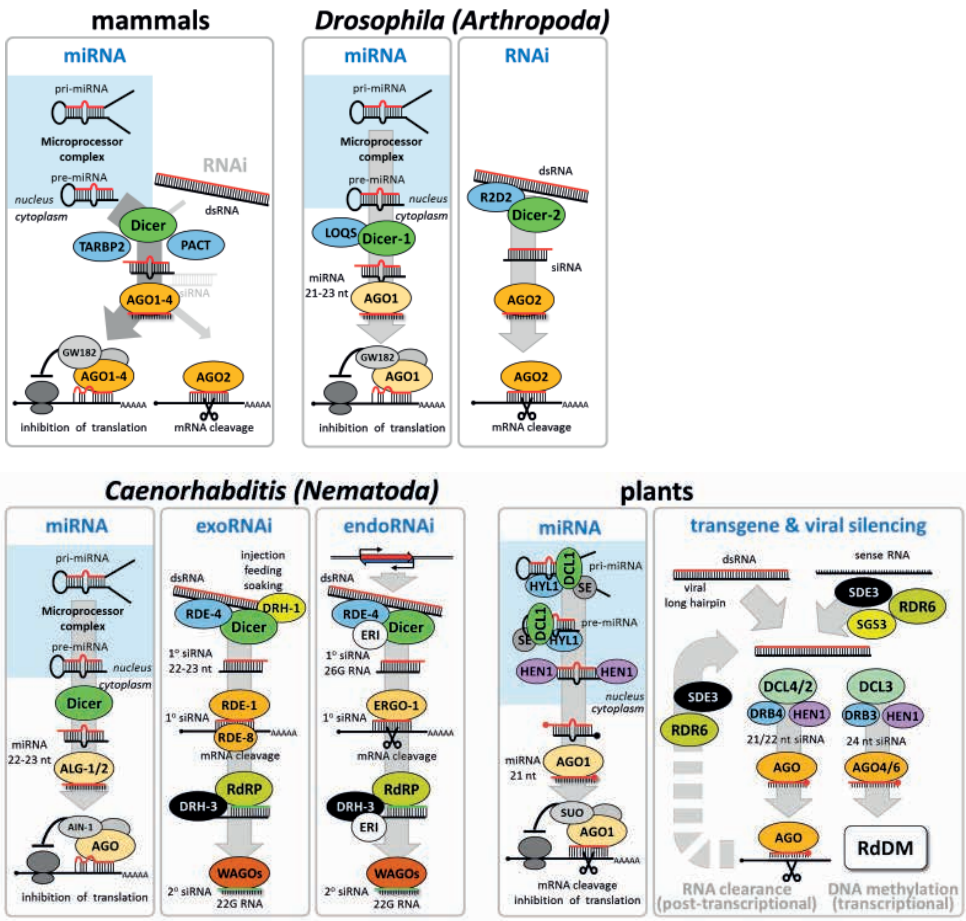

Figure 1 miRNA and RNAi pathways in animals and plants
The schemes depict key components of miRNA and RNAi pathways in the main eukaryotic model systems

In animals, the miRNA pathway, which is primarily a gene-regulating pathway, is highly conserved. The canonical miRNA biogenesis is a spatially separated into two-steps. The first step takes place in the nucleus where RNase III Drosha, a component of the Microprocessor complex, releases a precursor miRNA (pre-miRNA) from a primary miRNA transcript (pri-miRNA). Next, a pre-miRNA is transported to the cytoplasm where it is cleaved by a second RNase III Dicer. Dicer releases a miRNA duplex of which one strand will be loaded on an Argonaute protein. The miRNA pathway in plants operates similarly but employs only a single nuclear Dicer-like 1 (DCL1) RNase III to produce pre-miRNAs and miRNAs. The second important difference is the methylation of plant miRNAs at their 3' end mediated by HEN1 methyltransferase.

The RNAi pathway is much more diverse across animals and plants. It is conceivable given the antiviral role of RNAi where the parasite:host interactions can accelerate evolution of RNAi pathways in different taxons. Despite the differences, RNAi and miRNA pathways share common features, which include biogenesis of small RNAs involving Dicer and effector complexes containing an Argonaute protein carrying a small RNA. Argonaute proteins are composed of four main domains: the central PAZ domain, the C-terminal PIWI (P-element induced wimpy testis), the N-terminal domain, and the MID domain between PAZ and PIWI domains (Fig. 2). The PIWI domain has an RNase H-like fold and carries a "slicer" activity (Ma et al., 2005; Parker et al., 2004; Song et al., 2004; Yuan et al., 2005). Argonaute proteins fall into three distinct groups (reviewed in Faehnle and Joshua-Tor, 2007): (1) AGO proteins, found in all kingdoms, (2) PIWI proteins found in animals, and (3) WAGO proteins found only in nematodes.

From the mechanistic perspective, post-transcriptional repression by small RNAs employs two distinct yet related (and often overlapping) modes of action:

Direct endonucleolytic RNA cleavage mediated by the so-called "slicer" activity of an Argonaute protein. This mode of action needs two conditions to be met: (i) the Argonaute protein has the slicer activity (not all family members have it) and (ii) there needs to be extensive base pairing between the Argonaute-bound small RNA and the cognate RNA. Extensive base pairing positions the cognate RNA such that it can be sliced in the position corresponding the middle of the guiding small RNA. This mode of action has been traditionally associated with RNAi and will be refered to as "RNAi-like" targeting. However, it should be pointed out that the two conditions for RNA-like targeting do not exclude miRNAs and, in fact, it is well established that miRNAs loaded on a slicing Argonaute would guide slicing of perfectly complementary cognate RNAs.

Indirect mRNA destabilization, which is found when an Argonaute protein lacks the slicing activity or the base pairing is incomplete and prevents positioning of the cognate RNA (typically lack of base pairing in the middle of the small RNA:target RNA duplex. In these cases, Argonaute-bound small RNAs provide sufficiently stable interaction for target recognition while the repression is mediated by Argonaute-interacting partners. While the precise mode of action is still debated and may vary between different cell types and model systems, it seems to be coupled with common mechanisms of mRNA destabilization, i.e. deadenylation and decapping.

At least four types of RNAi & miRNA pathway combinations can be recognized in animals and plants (Fig. 1):

(I) overlapping miRNA and RNAi pathways with a single-set of Dicer and Argonaute proteins and without an RNA-dependent RNA polymerase (RdRP) – typical for vertebrates especially mammals. The molecular machinery in the cell primarily produces miRNAs but it can also support canonical RNAi, which can be observed under rare circumstances. The term RNAi in mammals is commonly used for RNAi-like cleavage mediated by a siRNA loaded on AGO2. However, siRNAs are being loaded on all four mammalian AGO protein (Meister et al., 2004), and once loaded, their behavior is indistinguishable from miRNAs. This functional overlap at the level of the effector complex is the major source of the so-called "off-targeting" phenomenon where siRNAs target also other mRNAs through miRNA-like mode of action.

(II) separated miRNA and RNAi pathways with dedicated Dicer and Argonaute proteins (no RdRPs). This arrangement is observed in Arthropods (*Drosophila*).

(III) distinct miRNA pathway and a complex RNAi system employing RdRp(s) sharing a single Dicer. This arrangement is observed in nematodes where expansion of Argonaute proteins created a highly complex RNA silencing system

(IV) separated miRNA pathway and a complex RNAi system employing RdRp(s) with multiple Dicer and Argonaute proteins. This arrangement is observed in plants.

Mechanistical aspects of target recognition and its specificity will be discussed next.

Small RNA:target RNA base pairing

A small RNA loaded on an Argonaute protein functions as a guide selectively recognizing cognate RNAs through sequence complementarity. Sequence complementarity can be high (full or almost full) or partial. High sequence complementarity operates in RNAi-mediated innate immunity and genome defense where it is desirable to degrade all nucleic acids with highly similar sequences. High sequence complementarity is also observed for many plant miRNAs, which could be, at least in part, a consequence of their evolution (Allen et al., 2004; Llave et al., 2002). Animal miRNAs and some plant miRNAs have typically partial sequence complementarity, which seems to be non-randomly distributed along a small RNA (reviewed, for example in Bartel, 2009). Partial complementarity could be seen as a minimal requirement for functional target recognition formed by natural selection. However,

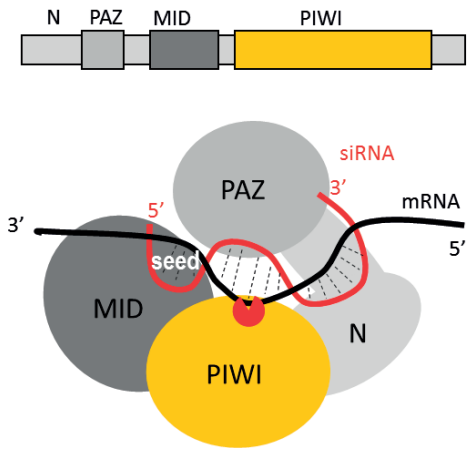

Figure 2 Argonaute protein structure
Schematic domain organization of an Argonaute protein. The scheme shows how a siRNA-loaded Argonaute cleaves a perfectly complementary RNA, which becomes accessible by the catalytical center in the PIWI domain upon base pairing with a small RNA. Nucleotides 2–8 of the small RNA initiate the interaction with the cognate RNA and form the so-called "seed", which has a highly predictive value for miRNA binding sites and siRNA off-targeting. The cognate mRNA is cleaved in the middle of the base paired sequence by the slicer activity depicted as a red pac-man.

before addressing small RNA:target RNA complementarity, I will review the structure of Argonaute proteins and its implications for base pairing and target recognition. The reason is that structural analyses of Argonaute proteins provided important insights into the mechanism of how an Argonaute-loaded small RNA recognizes and binds its target.

Structural insights into target recognition by Argonaute-bound small RNAs

The one of the fundamental steps in deciphering rules governing target recognition and repression in RNA silencing is understanding the structure of a cognate RNA bound to a guide RNA loaded on an Argonaute protein. The pioneering structural analysis of full-length Argonaute proteins has been carried out on crystalized archaeal proteins from *Pyrococcus furiosus* (Song et al., 2004), *Aquifex aeolicus* (Yuan et al., 2005), *Archaeglobus fulgidus* (Ma et al., 2005; Parker et al., 2005), and *Thermus thermophiles* (Wang et al., 2008; Wang et al., 2009) and, subsequently on human AGO1 and AGO2 proteins (Elkayam et al., 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2016; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014).

Structural analysis of archaeal proteins revealed that Argonaute proteins are composed of four main domains: the central PAZ domain, the C-terminal PIWI, the N-terminal domain, and the MID domain between PAZ and PIWI domains. A small RNA is anchored with its 3' end in the PAZ domain and the 5' end in a binding pocket between the MID domain and the PIWI domain (Fig. 2). Human AGO1 and AGO2 proteins also show this organization (Elkayam et al., 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2016; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014). While both proteins accommodate siRNAs and miRNAs, only AGO2 has the slicer activity (Liu et al., 2004; Meister et al., 2004). The crystal structure of human AGO2 revealed a bilobed molecule with a central cleft for binding guide and target RNAs (Elkayam et al., 2012; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014). The crystal structures of human AGO1 bound to endogenous co-purified RNAs or loaded with miRNA (let-7) are very similar to the structures of AGO2 despite the fact that AGO1 lacks the slicer activity (Faehnle et al., 2013; Nakanishi et al., 2013).

The key observation coming from the structural analysis is that nucleotides 2 to 6 of a guide RNA are positioned in an A-form conformation for base pairing with target messenger RNAs (Elkayam et al., 2012; Faehnle et al., 2013; Nakanishi et al., 2013; Schirle et al., 2016; Schirle and MacRae, 2012; Schirle et al., 2015; Schirle et al., 2014) (Fig. 3). An RNA molecule can occur in many three dimensional conformations because there are multiple angles along which it can rotate its parts. Accordingly, initiation of base pairing requires proper conformation of two RNA molecules in order to initiate formation of hydrogen bonds between two complementary molecules. An Argonaute protein facilitates base pairing between a small RNA and a complementary RNA (= target recognition) by exposing nucleotides 2–6 arranged in a conformation needed for proper base pairing.

Between nucleotides 6 and 7, there is a kink that may function in miRNA target recognition or release of sliced RNA products. (Schirle and MacRae, 2012). Crystallization of loaded human AGO2 in the presence of target RNA sequences suggested a stepwise mechanism for interaction with cognate RNAs. First, AGO2 exposes guide nucleotides (nt) 2 to

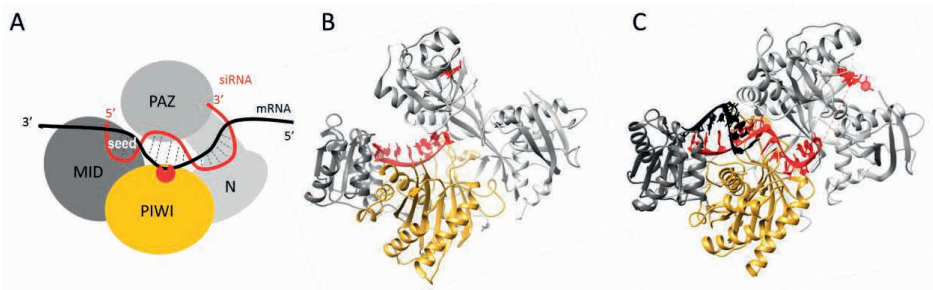

Figure 3 Crystal structures of Argonautes with bound RNAs
(A) A schematic depiction of AGO2 domain organization. (B) AGO2 with bound small RNA (in red), visible is the seed in A conformation (Schirle et al., 2014). (C) AGO2 loaded with a small RNA (in red) interacting with a target RNA (in black) (Schirle et al., 2014). Data for visualization were obtained from wwPDB and displayed in UCSF Chimera.

5 for initial target pairing, which then promotes conformational changes that expose nt 2 to 8 and 13 to 16 for further target recognition (Schirle et al., 2014). miRNA binding seem to lock the otherwise flexible AGO2 enzyme in a stable conformation (Elkayam et al., 2012). The structure of human Ago2 bound to miR-20a implies that the miRNA is anchored at both ends by the MID and PAZ domains with several kinks and turns along the binding groove (Elkayam et al., 2012). Spurious slicing of miRNA targets is avoided through an inhibitory coordination of one catalytic magnesium ion (Schirle et al., 2014). Evolutionary changes that rendered hAGO1 inactive included a mutation of a catalytic tetrad residue and mutations on a loop near the actives site (Faehnle et al., 2013; Nakanishi et al., 2013). Importantly, the PIWI domain contains tandem tryptophan-binding pockets, that function in recruitment of glycine-tryptophan-182 (GW182) or other tryptophan-rich cofactors (Schirle and MacRae, 2012). Computer simulation of the structural and functional dynamics of human AGO2 and the interaction mechanism with siRNAs confirmed that AGO2 adopts two conformations such as “open” and “close” and the PAZ is a highly flexible region. (Bhandare and Ramaswamy, 2016). Models of miRNA-loaded Argonautes imply that Argonautes adopt variable conformations at distinct target sites that generate distorted, imperfect miRNA-target duplexes where structural distortions are better tolerated in solvent-exposed seed and 3’-end regions than in the central duplex region (Gan and Gunsalus, 2015).

Structural analysis also clarified the effect of the first nucleotide in the cognate site, which does not base pair with the loaded small RNA because the first nucleotide of the small RNA (frequently U) is buried in the 5’ end-binding pocket. Yet, it was observed that interaction with the cognate site is enhanced by adenosine in the position 1 of a miRNA binding site; the structural analysis revealed that the adenosine in the mRNA is recognized indirectly by AGO2 through a hydrogen-bonding network of water molecules that preferentially interacts with the N6 amine on the adenine base (Schirle et al., 2015). Importantly, N6 adenosine methylation blocks recognition of the adenosine, which might reflect a possible mechanism for regulating of miRNA binding through covalent modification of miRNA binding sites (Schirle et al., 2015).

Figure 4 Small RNA domains.
Small RNAs loaded onto AGO proteins can be divided into modules including the 5' the anchor, seed sequence, central part, 3' supplementary sequence, and tail (Wee et al., 2012).

These data provide structural foundations of many features of target recognition and can be used for computer simulations of miRNA-target interaction in the context of the loaded Argonaute structure. In fact, an algorithm MiREN, which builds and scores three-dimensional models of the ternary complex formed by AGO, a miRNA and 22 nt of a target mRNA, can be used to assess the likelihood that an RNA molecule is the target of a given miRNA (Leoni and Tramontano, 2016).

Importantly, they also explain features associated with different regions of miRNA and siRNA sequences that were identified in kinetic and bioinformatics studies. Taken together, crystal structures of AGO2 explain the nucleotide-pairing patterns that emerged during previous studies of miRNA sequences, namely analyses of conservations of miRNA binding sites and biochemical analyses of target recognition, which are discussed later.

small RNA:target RNA base pairing

Sequence complementarity between a small RNA and its target RNA can be full (or almost full) or partial. Full complementarity is typically associated with siRNAs while partial with miRNAs although imperfect base pairing of siRNAs and perfect base pairing of miRNAs also occur. To provide a framework for this section, I first review the full-complementarity, then the partial complementarity involving base pairing of 5' small RNA nucleotides (the seed, Fig. 4) and then seedless (non-canonical, non-seed) interactions and their implications on target recognition, prediction and effective repression. Importantly, target mRNAs are as efficiently repressed by microRNA-binding sites in the 5 ' UTR as in the 3 ' UTR as shown in experiments in cultured human cells (Lytle et al., 2007).

siRNA complementarity and sequence features

RNAi efficiency correlates well with the binding energy of a siRNA to its mRNA target (Muckstein et al., 2006). While full complementarity yields a perfect duplex in which all nucleotides participate seemingly equally, some positive correlations were identified between positions of specific nucleotides and siRNA suppressing efficiency. These features may reflect positive effects on Argonaute loading (strand selection) as well as on target recognition. Analysis of the efficiency of ~600 siRNAs suggested higher siRNA efficiency with A/U at positions 10 and 19, a G/C at position 1, and more than three A/Us between positions 13 and 19, in the sense strand of the siRNA sequence (Jagla et al., 2005). Furthermore, specific residues at every third position of an siRNA influence its efficient RNAi

activity, which might reflect interaction with TARBP2 during formation of the RNA-induced silencing complex (RISC) (Katoh and Suzuki, 2007).

Target recognition by siRNAs is highly specific. However, discrimination of RNAi between two sequences differing by a single nucleotide varies according to the position of the mismatch. A systematic analysis of single-nucleotide mutations in target sites of a functionally validated siRNA showed that the position of the mismatched base pair and the identity of the nucleotides forming the mismatch matter for effective silencing (Du et al., 2005). A:C mismatches were, in addition to the G:U wobble base pairs, surprisingly well tolerated and target sites containing such mismatches were silenced almost as efficiently as with full complementarity (Du et al., 2005). G:U wobble base pairing in the central part of the antisense strand caused a pronounced decrease in activity, while mutations at the 5' and 3'ends were well-tolerated (Holen et al., 2005). Interestingly, analysis of siRNA selectivity suggested that siRNAs with G:U wobble base pairs or a mismatches located in the "seed" are discriminating less between perfect and mismatched target than those in which the mismatch was located 3' to the seed (nucleotides 9–14); this region is critical for target cleavage but not siRNA binding (Schwarz et al., 2006).

miRNAs with extensive base pairing

Target recognition by miRNAs in plants is commonly thought to involve extensive base pairing and RNAi-like cleavage of the target (reviewed in Axtell, 2013; Wang et al., 2015). This notion stems from the perfect complementarity between miR171 and its SCARECROW-LIKE (SCL) mRNA target, which was the first identified miRNA:mRNA interaction in plants (Llave et al., 2002). However, the perfect complementarity is rather an exception as most of the identified miRNA targets in plant cells have some imperfect base pairing (summarized in (Jones-Rhoades and Bartel, 2004; Jones-Rhoades et al., 2006)). Extensive base pairing and microRNA-directed RNAi-like cleavage exists also in animals but it is rare; one of the exceptional cases is HOXB8 mRNA cleavage by miR-196 (Yekta et al., 2004)

Mismatches to the miRNA 5' regions strongly reduce repression but are found in several natural miRNA-binding sites while miRNA binding with a few mismatches to the miRNA 3' regions are common in plants and are often equally (or even more) effective as perfectly matched sites (Liu et al., 2014b). Central mismatches interfere with repression (Liu et al., 2014b). However, miR398 in *Arabidopsis* binds 5'UTR of the blue copper-binding protein mRNA with a bulge of six nucleotides opposite to the 5' region of the miRNA (Brousse et al., 2014). These and other studies led to consensus base pairing rules for a functional plant miRNA-target interaction: little tolerance for mismatches at positions 2–13, with especially little tolerance of mismatches at positions 9–11, and more tolerance of mismatches at positions 1, and 14–21 (Wang et al., 2015). This is in contrast with animal miRNAs where pairing at positions 2–7 can be sufficient for a functional interaction (Bartel, 2009).

High sequence complementarity in mammals may be coupled with Argonaute "unloading". It was found that highly complementary target RNAs significantly accelerate release of the guide RNA from Ago2. Unloading can be enhanced by mismatches between the

target and miRNA's 5' end and attenuated by mismatches to miRNA's 3' end (De et al., 2013).

Imperfect base pairing of miRNAs

Animal miRNAs typically base pair imperfectly with their targets. Target sites can be grouped into two broad categories. 5' dominant sites have sufficient complementarity to the miRNA 5' end to function with little or no support from pairing to the miRNA 3' end. Indeed, sites with 3' pairing below the random noise level are functional given a strong 5' end. In contrast, 3' compensatory sites have insufficient 5' pairing and require strong 3' pairing for function (Brennecke et al., 2005). Accordingly, I will separately discuss the canonical base pairing involving miRNA's 5' end (the seed) and the non-canonical (seedless) interactions. I will start with the canonical interaction involving base pairing of the seed because it is the most studied and integrates knowledge from structural studies as well as sequence analyses.

Seed-involving interactions

The seed sequence concept emerged already during pioneering work on miRNA annotation where it became apparent that miRNAs form families sharing 5' sequences (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Then it became clear that the seed sequence is a strong predictor for miRNA targets (Lewis et al., 2005; Sood et al., 2006) as well as for siRNA off-targeting (Jackson et al., 2006b). An analysis of more than 18,000 high-confidence miRNA-mRNA interactions suggested that binding of most miRNAs includes the 5' seed region, while around 60% of seed interactions contained bulged or mismatched nucleotides (Helwak et al., 2013). The molecular mechanism of miRNA and target recognition (reviewed in Bartel, 2009) provides an explanation for the significance of the seed sequence and, while there are also small RNA:target mRNA interactions that do not involve the seed sequence, the concept of the seed is sufficient to explain that any AGO-loaded small RNA in any cell type has the potential to interact with hundreds and thousands of different mRNAs. In fact the estimates for human mRNAs targeted by miRNAs are between 30 and >60% (Friedman et al., 2009; Lewis et al., 2005).

The seed region is generally defined as a 7nt region mapping to positions 2–8 and it strongly confers specificities of animal miRNAs to their mRNA targets. There is a high functional cost of even single nucleotide changes within seed regions, which is consistent with their high sequence conservation among miRNA families both within and between species and suggests processes that may underlie the evolution of miRNA regulatory control (Hill et al., 2014). The target specificity determined by the seed has evolutionary and biological implications because single nucleotide polymorphisms in canonical miRNA binding sites would affect miRNA-mediated regulations, a notion supported also by experimental data (Afonso-Grunz and Muller, 2015; Vosa et al., 2015).

The canonical 7nt seed can be divided into several types (Ellwanger et al., 2011). More specifically, the core seeds have been described as a 6-mer (bases 2–7), 7-mer ("7-mer-A1" being bases 1–7, and "7-mer-m8" being bases 2–8), and 8-mer (bases 1–8); sometimes the

7-mer-A1 and 8-mer seeds are required to have an adenine, 'A', as the first nucleotide types (Bartel, 2009; Ellwanger et al., 2011).

Longer seeds, i.e. seeds of 7 or 8 nucleotides in length are more evolutionarily conserved than shorter ones (Ellwanger et al., 2011). Longer seeds confer higher specificity and repression. It was reported that the extent of the seed match has a strong impact on resulting target repression: single 8 mer seed match mediates down-regulation comparable to two 7 mer seed matches (Nielsen et al., 2007). However, others did not observe a linear relationship between seed length and miRNA expression dysregulation, which does not support the hypothesis the seed region length alone influences mRNA repression. (Mullany et al., 2016)

In any case, the majority of functional target sites seems formed by less specific seeds of only 6 nt indicating a crucial biological role of this type (Ellwanger et al., 2011). In fact, pairing at positions 2–7 is sufficient for a functional interaction of animal miRNAs with their targets (Bartel, 2009). In contrast, seed pairing does not appear to be critical for land plant miRNAs (Liu et al., 2014b).

The minimal requirement for miRNA:mRNA interactions in animals explains the large numbers of targets of animal miRNAs and the fact that, the majority of functional sites is poorly detected by common prediction methods (Ellwanger et al., 2011). While the initial studies suggested that average miRNAs have approximately 100 target sites (Brennecke et al., 2005), subsequent bioinformatics and experimental identification of miRNA targets suggest even higher number of target sites.

There are several targeting determinants that enhance seed match-associated mRNA repression, including the presence of adenosine opposite miRNA base 1 (this functionality is explained by Argonaute protein structure (Schirle et al., 2015)) and of adenosine or uridine opposite miRNA base 9, independent of complementarity to the siRNA/miRNA (Lewis et al., 2005; Nielsen et al., 2007). Furthermore, seed-based canonical target recognition was dependent on the GC content of the miRNA seed – low GC content in the seed was coupled with non-canonical target recognition. (Wang, 2014). Additional reported determinants beyond seed pairing include: AU-rich nucleotide composition near the site, proximity to sites for co-expressed miRNAs (which leads to cooperative action), proximity to residues pairing to miRNA nucleotides 13–16, positioning within the 3'UTR at least 15 nt from the stop codon, and positioning away from the center of long UTRs (Grimson et al., 2007).

Non-canonical – non-seed interactions

There is large variety of miRNA-target duplex structures, which include seedless interactions (reviewed in Cipolla, 2014; Seok et al., 2016a). The existence of seedless interactions explains reports that perfect seed pairing is not a generally reliable predictor for miRNA-target interactions (Didiano and Hobert, 2006). Despite attempts to classify non-canonical interactions (Xu et al., 2014b) and tertiary structure-based modelling of miRNA interactions (Gan and Gunsalus, 2015), bioinformatic prediction of non-canonical interactions is far from ideal. A solution is integration of bioinformatic target prediction with biochemically identified miRNA binding sites. Such analyses suggested that most miRNA targets were of a non-canonical type, i.e. not involving perfect complementarity in the seed region

(Khorshid et al., 2013; Wang, 2014). Importantly, analysis of AGO-associated mRNAs that lack seed complementarity with miRNAs suggested that AGO might have its own binding preference within target mRNAs, independent of guide miRNAs (Li et al., 2014). A structurally accessible and evolutionarily conserved region (~10 nucleotides in length) was identified that alone can accurately predict AGO-mRNA associations, independent of the presence of miRNA binding sites (Li et al., 2014). In any case, the impact of non-canonical targeting regarding target downregulation is not fully resolved (Khorshid et al., 2013; Martin et al., 2014; Wang, 2014).

Other important factors influencing target recognition and repression

It is important to recognize that sequence complementarity between a small RNA and its putative target is not sufficient to make any prediction about silencing of the target because there are other important factors at play. The two most important are discussed in the next two sections are (I) the binding site accessibility and (II) stoichiometry between a small RNA ant its target (or binding kinetics). Other factors, which might contribute to silencing in a context-dependent manner are, for example, alternative polyadenylation and arrangement of miRNA binding sites in 3'UTRs might cause different effects in different cells (Hon and Zhang, 2007; Majoros and Ohler, 2007; Nam et al., 2014a). In particular, there was a strong preference reported for targets to be located in close vicinity of the stop codon and the polyadenylation sites. (Majoros and Ohler, 2007).

Binding site accessibility

Mere sequence complementarity is not a sufficient predictor whether base pairing will occur *in vivo*. RNA molecules always form secondary structures and, in the cellular context, a number of proteins interacts with RNA molecules. Accordingly, secondary structures or RNA binding proteins may prevent base pairing of two complementary sequences. The issue of sequence accessibility was recognized during early RNAi experiments with stochastic knockdown efficiency. When searching for factors influencing knock-down efficiency, attention turned to the local RNA structure at siRNA target sites and it was demonstrated that local RNA target structure is an important factor for siRNA efficacy (Schubert et al., 2005). Accordingly, siRNA design tools started to accommodate not only properties of siRNAs but also properties of the target site because it strongly increased efficiency of designed siRNAs (Heale et al., 2005, 2006; Shao et al., 2007; Tafer et al., 2008).

Systematic investigation of siRNA:target RNA interactions and the effect of local secondary structures provided also insights into the molecular mechanism of target recognition. It was shown in vitro and in vivo, that the accessibility of the target site correlates directly with the efficiency of cleavage, demonstrating that RISC is not unfolding structured RNA (Ameres et al., 2007). During target recognition, RISC transiently contacts single-stranded RNA nonspecifically and promotes siRNA-target RNA annealing (Ameres et al., 2007).The seed of Argonaute-associated siRNA creates a thermodynamic threshold that determines the stable association of RISC and the target RNA (Ameres et al., 2007).

The same principles apparently apply for miRNA-mediated repression (Long et al., 2007; Xu et al., 2014b). Mutations diminishing target accessibility substantially reduce microRNA-mediated translational repression, with effects comparable to those of mutations that disrupt sequence complementarity (Kertesz et al., 2007).

small RNA:target RNA stoichiometry and binding kinetics

The second critical factor for target repression is stoichiometry between a small RNA ant its target. This is especially important for the miRNA-like type of target repression because a miRNA must remain associated with its target RNA in order to induce its translational repression and degradation. Thus, suppression of a specific mRNA by a miRNA requires enough miRNA molecules that would assure enough interactions with binding sites in that particular RNA while these binding sites essentially compete with all binding sites for that miRNA in the transcriptome.

Biochemical analyses of stoichiometry and kinetics

Kinetic data should be taken as a biochemical range for any hypotheses concerning target recognition and biological effects of small RNAs in the context of loaded RISC. Among these is a detailed kinetic study of *Drosophila* and mouse AGO2 RISCs (Wee et al., 2012).

It was shown that siRNA-programmed RISC is a classical Michaelis-Menten enzyme in the presence of ATP (Haley and Zamore, 2004). In the absence of ATP, the rate of multiple rounds of catalysis is limited by release of the cleaved products (Haley and Zamore, 2004). Kinetic analysis suggests that different regions of the siRNA play distinct roles in the cycle of target recognition, cleavage, and product release (Haley and Zamore, 2004). Later, it was shown that Argonaute divides its RNA guide into domains with distinct functions and RNA-binding properties. (Wee et al., 2012) According to this analysis, small RNAs loaded onto AGO proteins are actually composed of five distinct modules the anchor, seed, central, 3' supplementary, and tail (Fig. 4) (Wee et al., 2012). Bases near the siRNA 5' end disproportionately contribute to target RNA-binding energy, whereas base pairs formed by the central and 3' regions of the siRNA provide a helical geometry required for catalysis (Haley and Zamore, 2004). Mouse AGO2, which mainly mediates miRNA-directed repression in vivo, dissociates rapidly and with similar rates for fully paired and seed-matched targets (Wee et al., 2012). An important conclusion from this study is that low-abundant miRNAs are unlikely to contribute much biologically meaningful regulation because they are present at a concentration less than their KD for seed-matching targets, which are in a picomolar range (Wee et al., 2012). Another study characterized siRNA binding, target RNA recognition, sequence-specific cleavage and product release by recombinant human Ago 2 (hAgo2). This yielded a minimal mechanistic model describing fundamental steps during RNAi, which is consistent with a "two-state" model of RISC action (Deerberg et al., 2013). Finally, it was found that Mg2+ concentration, influences AGO2 structural flexibility and is important for its catalytic/functional activity, with low [Mg2+] favoring greater Ago2 flexibility (e.g., greater entropy) and less miRNA/mRNA duplex stability, thus favoring slicing(Nam et al., 2014b).

Importantly, it seems that miRNA:mRNA stoichiometry cannot be simply determined by quantifying RNAs. Quantification of Argonaute-associated endogenous miRNAs or exogenous siRNAs in cultured cells suggested that only a small proportion (even <10%) of such small RNAs is loaded on Argonautes (Janas et al., 2012; Stalder et al., 2013). Furthermore, a substantial percentage of the miRNA pool associated with mRNAs without Argonautes (Janas et al., 2012; Stalder et al., 2013). It was also found that endogenous human miRNAs vary widely, by >100-fold, in their level of RISC association and show that the level of Ago binding is a better indicator of inhibitory potential than is the total level of miRNA expression (Flores et al., 2014). Together, these data indicate that the level of RISC association of a given endogenous miRNA is regulated by the available RNA targetome and predicts miRNA function. (Flores et al., 2014).

Small RNA:target RNA binding single-molecule analysis

Recent advances in single-molecule analysis brought also single- molecule data about RISC:target interaction, which is consistent with other biochemical data and the two state model for Argonaute action (Li and Zhang, 2012; Zander et al., 2014).

Loaded AGO2 utilizes short RNAs as specificity determinants with thermodynamic and kinetic properties more typical of RNA-binding proteins. A small RNA loaded on Argonaute does not follows rules by which free oligonucleotides find, bind, and dissociate from complementary nucleic acid sequences (Salomon et al., 2015). This is conceivable given the fixed "A" conformation of the seed of a small RNA loaded on an Argonaute protein.

Single-molecule fluorescence experiments using a minimal RISC (a small RNA and AGO2) showed that target binding starts at the seed region of the guide RNA (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). AGO2 initially scans for target sites with complementarity to nucleotides 2–4 of the miRNA. This initial transient interaction propagates into a stable association when target complementarity extends to nucleotides 2–8. This stepwise recognition process is coupled to lateral diffusion of AGO2 along the target RNA, which promotes the target search by enhancing the retention of AGO2 on the RNA (Chandradoss et al., 2015). Stable RISC binding is thus efficiently established with the seed match only, providing a potential explanation for the seed-match rule of miRNA target selection (Chandradoss et al., 2015; Jo et al., 2015a; Jo et al., 2015b). Mouse AGO2 binds tighter to miRNA targets than its RNAi cleavage product, even though the cleaved product contains more base pairs (Salomon et al., 2015). Annealing between miRNA and its target with poor seed match proceeds in a stepwise way, which is in accordance with the increase in the number of conformational states of miRNA-target duplex accommodated by the miRISC, suggesting the structural plasticity of human miRISC to conciliate the mismatches in seed region (Li and Zhang, 2012)

Target cleavage required extensive sequence complementarity and accelerated core-RISC dissociation for recycling (Jo et al., 2015a) and sensitively depended on the sequence (Jo et al., 2015b). While RISC generally releases the 5' cleavage fragment from the guide 3' supplementary region first and then the 3' fragment from the seed region. This order can be reversed by extreme stabilization of the 3' supplementary region or mismatches in the seed region. Therefore, the release order of the two cleavage fragments is influenced by the

stability in each region, in contrast to the unidirectional base pairing propagation from the seed to the 3' supplementary region upon target recognition.(Yao et al., 2015).

Off-targeting – causes and remedies

Off-targeting effects surfaced as a major issue in RNAi experiments when the effects of RNAi treatment were systematically analyzed (Fedorov et al., 2006; Jackson et al., 2003; Lin et al., 2005; Scacheri et al., 2004; Snove and Holen, 2004). One of the most revealing data came from mammalian cells transfected with different siRNAs targeting the same gene, which were systematically analyzed using microarrays (Jackson et al., 2003). Using 16 different siRNAs against IGF1R and 8 different siRNAs against MAPK14, strong siRNA-specific expression changes were found in transfected cells with only a few genes regulated in common by siRNAs targeting the same gene. Off-targeting effects were also found also in other animal models (Ma et al., 2006) and plants (Xu et al., 2006). In fact, off-targeting causes a significant bias in high-throughput RNAi screens (Ma et al., 2006)

Off-targeting is concentration dependent, it could be attributed to both siRNA strands, and a portion of off-targeting appears to be caused by partial complementarity between a siRNA and its target, reminiscent of the 5' seed regions of miRNAs (Aleman et al., 2007; Birmingham et al., 2006; Jackson et al., 2003; Jackson et al., 2006b; Qiu et al., 2007). In some cases of off-targeting, no correlation between predicted and actual off-target effects was reported (Hanning et al., 2013). However, this probably reflects problems of accurate miRNA target prediction rather than the absence of miRNA-like off-targeting. In any case, a recent systematic analysis of off-targeting effects confirmed that strength of base pairing in the siRNA seed region is the primary factor determining the efficiency of off-target silencing (Kamola et al., 2015)

The main cause of off-targeting is miRNA-like behavior of siRNAs. It was experimentally demonstrated in mammalian cells that siRNAs can function as miRNAs (Doench et al., 2003) and that siRNAs imperfectly matching endogenous mRNAs repress translation (Martin and Caplen, 2006; Saxena et al., 2003) suggesting that miRNAs and siRNAs use similar if not identical, mechanisms for target repression (Zeng et al., 2003). The current view of mammalian RNAi is that experimental RNAi induced with a siRNA or shRNA hijacks the molecular machinery dedicated to the miRNA pathway (reviewed in Svoboda, 2014). Consequently, some degree of off-targeting likely occurs in every RNAi experiment.

Importantly, experimental RNAi can also cause artifacts through saturation of the miRNA pathway, which essentially suppresses normal miRNA function (Khan et al., 2009). Exportin 5 seems to be a bottleneck for an effective RNA silencing (Lu and Cullen, 2004; Yi et al., 2005). Indeed, lethal non-specific effects observed with type I shRNAs delivered to the mouse liver by a viral vector were linked to the saturation of Exportin 5 (Grimm et al., 2006). Inhibition of Exportin 5 could also provide an explanation to early lethality defects observed during generation transgenic mice carrying class I shRNA expression cassette (Cao et al., 2005).

Suppression and by-passing off-targeting

Off-targeting has been a recurring problem with RNAi experiments, especially in RNAi screens searching for novel regulators. Off-targeting was frequently causing false-positive results in such screens although this issue has been partially remedied (reviewed in Mohr et al., 2014; Petri and Meister, 2013). Below, I list options for dealing with off-targeting, which emerged from the literature review.

Appropriate experimental design

This is actually a simple solution, which emerged from initial experiments detecting off-targeting (reviewed in(Svoboda, 2007), which suggested that off-targeting operates through miRNA-like behavior of siRNAs and is concentration-dependent. Thus, a proper practice is to use the minimal effective siRNA concentration. Importantly, this step strongly reduces off-targeting but it does not eliminate it as the targeting siRNA is still present and functions as a miRNA (Jackson et al., 2003; Jackson et al., 2006b).

Pools of siRNA

An extension of a strategy to lower siRNA concentration to the point that off-targeting effects in the model system become very low or even undetectable. If a pool of 10 siRNAs is used at the same total siRNA concentration, a single siRNA is having ten times lower concentration and causes lower off-targeting effects. One can produce an siRNA pool by an enzymatic digest of long dsRNA with Dicer or simply purchase a number of siRNAs targeting a single mRNA. In fact, some companies offer pre-made siRNA pools. A unique type of siRNA pools are siPools, which are produced by in vitro transcription of tandemly arrayed siRNA sequences (Hannus et al., 2014)

Bioinformatics filtering

Since the siRNA seed region is strongly associated with off-target silencing (Jackson et al., 2006b; Kamola et al., 2015), it could be used to filter RNAi screening data to reduce of off-target rates (Yilmazel et al., 2014; Zhong et al., 2014). In fact, revised analysis of RNAi screens could identify functionally relevant genes suppressed by off-targeting (Adams et al., 2015; Lin et al., 2007; Singh et al., 2015).

Better small RNA design

Understanding of the molecular mechanism of RNAi is also reflected in constantly improving siRNA design which aims at providing siRNAs specifically silencing a gene of interest with little or no off-target effects and no cell toxicity (reviewed in Ahmed et al., 2015; Tafer, 2014). Improved siRNA design can reduce off-targeting in several ways. First, siRNAs designed for efficient strand selection would have reduced off-targeting caused by AGO-loaded passenger strand. It was also found that increased siRNA duplex stability

correlates with reduced off-target and elevated on-target effects (Petri et al., 2011). This can be, for example, influenced by the seed binding energy and seed composition, which would determine the pool of potential binding sites in the transcriptome and the difference between on-target and off-target RNAs (Das et al., 2013a; Das et al., 2013b). Adaptations of siRNA/shRNA design to reduce off-target effects include weak base pairing in both seed and 3 ‘ regions (Gu et al., 2014) and evaluation of potential cross-hybridization candidates (Anderson et al., 2008; Yamada and Morishita, 2005). Reduced off-targeting features were subsequently integrated into siRNA design tools such as siDirect (Naito and Ui-Tei, 2013; Naito et al., 2009).

Mismatch introduction

Mismatch introduction into siRNA at the positions 2 of the base pairing also weakens off-targeting (Dua et al., 2011; Li et al., 2015)

Chemical modifications of small RNAs

The discovery that off-targeting involves miRNA-like behavior of siRNAs prompted research on chemical modifications that would reduce miRNA-like behavior while not interfering with desired RNAi effects (Chiu and Rana, 2003). A thorough review of the chemical modifications is beyond the scope of this report but can be found elsewhere (Engels, 2013; Nolte et al., 2013; Peacock et al., 2011; Snove and Rossi, 2006). There are two common strategies, to reduce off-targeting – (I) Chemical modifications on the passenger strand preventing its loading, hence eliminating off-targeting caused by the passenger strand (Chen et al., 2008; Snead et al., 2013) and (II) Chemical modifications in the seed region, which interfere with miRNA-like target recognition but do not prevent specific RNAi targeting. Different chemistry was used for chemical modification of siRNAs with reduced off-targeting effects including unlocked nucleic acid (UNA) modification (Snead et al., 2013), locked nucleic acid (LNA) modification (Fluiter et al., 2009), 2’-O-methyl ribosyl (Chen et al., 2008; Jackson et al., 2006a), or abasic nucleotides (Seok et al., 2016b).

Position of the modification on the guiding strand is important for reduced off-targeting. While it is usually involving seed, the modified nucleotide may vary. For example, 2’-O-methyl ribosyl substitution at position 2 in the guide strand reduces most off-target effects caused by complementarity to the seed region of the siRNA guide strand (Jackson et al., 2006a). At the same time, an abasic nucleotide at the position 6 in the guide strand also eliminates miRNA-like off-target repression but preserves near-perfect on-target activity (80–100%) (Seok et al., 2016b).

Discerning specific RNAi phenotypes from off-targeting effects

While strategies for suppressing off-targeting effects clearly reduce experimental artifacts, off-targeting should be seen as a type of noise in RNAi experiments that cannot be completely eliminated. Assuming that some off-targeting occurs in every RNAi experiment, one can focus on a more important issue: how to identify biologically relevant effects

of off-targeting (phenotype) and separate them from the specific RNAi effect caused by knock-down of the desired gene. The idea is simple – while one can try to minimize off-targeting effects, the risk cannot be completely eliminated. Thus, it is equally important to use an appropriate experimental design, which allows to distinguish between off-targeting and specific RNAi effects. The two possible strategies were proposed a decade ago and were named "the two R's": rescue and redundancy (Echeverri et al., 2006).

The principle of the rescue strategy is expressing an RNAi-resistant version of the targeted gene. If a phenotype is caused by the gene knock-down, it should be rescued. It can be either mutated such that the base pairing with a short RNA is eliminated. One can, for example target 3'UTR and use a different one in the rescue construct or mutate/degenerate appropriate codon positions if targeting CDS. This strategy is powerful because it accepts all effects in an RNAi experiment and tests the contribution of the specific gene knock-down. For recent information on design of the rescue system see, for example, (Kumar, 2015)

The second strategy is based on phenotype redundancy. Two or more RNAi triggers with different sequences (i.e. specific siRNAs or shRNAs) producing the same phenotype decrease the probability that a phenotype would be caused by off-targeting. However, some common phenotypes (e.g. slower growth, apoptosis, and developmental arrest) may be a frequent off-targeting phenotype induced by different RNAi triggers, so the redundancy strategy would be less powerful than the rescue strategy described above. However, for some purposes (e.g. high-throughput RNAi screening), it might be easier to implement the redundancy strategy as a control for off-targeting than the rescue strategy.

Importantly, "non-targeting" controls (e.g. siRNAs with a random sequence or targeting non-expressed genes such as EGFP or luciferase) cannot be used controls for off-targeting for reasons mentioned above. It is a frequent misconception ignoring the fact that off-targeting is individual to each RNAi trigger because it is sequence-specific. "Non-targeting" siRNAs or shRNAs RNAs may serve as controls for the sequence-independent effects, such as interferon response and saturation of RNA silencing with an excess of exogenous short RNAs. If a small RNA is needed as a control for off-targeting, one may only use a pool of scrambled small RNAs, which would have highly diluted off-targeting effects.

Target identification – in silico & experimental approaches

Target identification is a common issue in the small RNA field (reviewed for example in Pasquinelli, 2012; Tarang and Weston, 2014). Target identification can utilize bioinformatic analysis, experimental analysis or their combination. Briefly, bioinformatic analysis primarily implements the canonical seed-match model, evolutionary conservation, and binding energy, which are often complemented by neural networks trained on sets of experimental data in order to optimize filtering parameters. Some bioinformatics tools also consider non-canonical binding sites. Importantly, a mere presence of a miRNA binding site is insufficient for predicting target regulation as additional factors influence the regulation, including the above-mentioned accessibility of a binding site and stoichiometry between a miRNA and its targets. In the end, experimental verification of microRNA targets is essential, prediction alone is insufficient (Law et al., 2013).

Target prediction in silico

Identification of many miRNAs in model organisms prompted development of bioinformatics tools for prediction of targeted mRNAs (Enright et al., 2003; Grun et al., 2005; John et al., 2004; Kiriakidou et al., 2004; Krek et al., 2005; Lewis et al., 2003; Stark et al., 2003). A number of bioinformatics tools emerged for miRNA analysis and target prediction (a comprehensive overview of all miRNA analysis tools is provided at https://tools4mirs.org/, for recent reviews on bioinformatic target prediction see, for example, (Elton and Yalowich, 2015; Lagana, 2015; Li and Zhang, 2015; Ristevski, 2015). A searchable database of systematically annotated miRNA tools can be found here: https://tools4mirs.org/software/target_prediction/.

Importantly, accurate bioinformatic prediction of miRNA-mediated repression is still problematic. This was shown, for example, during experiments with systematically generated artificial miRNAs targeting a desired gene (Arroyo et al., 2014). It turned out that seed-based artificial miRNA design was highly inefficient, as the majority of miRNAs with even perfect seed matches did not repress either target. Moreover, commonly used target prediction programs had problems to discriminate effective artificial miRNAs from ineffective ones, indicating that current algorithms do not fully accommodate important miRNA features allowing for designing artificial miRNAs (Arroyo et al., 2014). Another unresolved issue is reliable prediction of non-canonical (non-seed) miRNA binding sites as most algorithms are based on detection of seed-based miRNA binding sites.

Common target prediction tools for animal miRNAs

Among the prediction tools, several can be highlighted. These include Targetscan, miRanda, DIANA-microT, PicTAR, whose predictions were integrated into the miRBase, the central annotation database for miRNAs (Kozomara and Griffiths-Jones, 2014) and were also repeatedly evaluated in benchmark studies (Alexiou et al., 2009; Ding et al., 2012; Majoros et al., 2013; Peterson et al., 2014; Xu et al., 2014a). I add to these also MIRZA as it is one of the most recent algorithms, which in many aspects outperforms the other ones (Gumienny and Zavolan, 2015).

Targetscan (http://www.targetscan.org/vert_71/)

Targetscan is one of the most popular miRNA target prediction tools and its predictions are integrated in the miRBase (Kozomara and Griffiths-Jones, 2014). It is being developed in David Bartel's laboratory as a tool for miRNA target prediction for over a decade (Lewis et al., 2005; Lewis et al., 2003). It predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA but there is also an optional search for poorly conserved sites. Its development included also scoring for binding sites with mismatches in the seed region that are compensated by 3' end pairing (Friedman et al., 2009) an improved quantitative model of canonical targeting (Agarwal et al., 2015) and addition other features. The current version considers a site type and fourteen other features and, according to authors,

it outperforms other tools and matches high-throughput in vivo crosslinking approaches (Agarwal et al., 2015).

DIANA-MicroT (http://diana.imis.athena-innovation.gr/DianaTools/index.php)

DIANA-MicroT target prediction tools are another popular source for miRNA target prediction whose predictions are integrated with miRBase (Kozomara and Griffiths-Jones, 2014). DIANA-MicroT tools are being developed in Artemis Hatzigeorgiou's laboratory for over a decade (Alexiou et al., 2010; Kiriakidou et al., 2004; Maragkakis et al., 2009; Maragkakis et al., 2011; Megraw et al., 2007; Paraskevopoulou et al., 2013a; Paraskevopoulou et al., 2013b; Paraskevopoulou et al., 2016; Reczko et al., 2011; Sethupathy et al., 2006; Vergoulis et al., 2012; Vlachos et al., 2012). MicroT is specifically trained on a positive and a negative set of miRNA binding sites located in 3'-UTR and CDS regions. DIANA Tools offer target prediction algorithms (microT v4 and microT-CDS), databases of experimentally verified miRNA targets on coding and non-coding RNAs (TarBase v7.0 and LncBase), and tools for assessment of biological impacts of miRNAs (mirPath). In addition, the Web Server (v5.0) supports workflows enabling to perform complex functional miRNA analyses.

Pictar (http://www.pictar.org/)

Pictar is an algorithm for the identification of microRNA targets from Nikolaus Rajewsky's laboratory (Grun et al., 2005; Krek et al., 2005). Its predictions are also integrated with miRBase (Kozomara and Griffiths-Jones, 2014). Pictar offers for searching of targets of annotated miRNAs or mRNAs. Pictar predicts targets based on complementarity in a 7nt seed region, takes into account conservation and uses hidden Markov model approach to produce the final score. In contrast to Targetscan and DIANA-MicroT, Pictar has not been intensely developed. While it represents one of the older and simpler target prediction algorithms it is quite accurate prediction tool (Alexiou et al., 2009).

miRanda at microRNA.org – Targets and Expression (http://www.microrna.org/)

miRanda belongs among the pioneering target prediction algorithms (Enright et al., 2003; John et al., 2004). Its latest version miRanda-miRSVR (Betel et al., 2010; Betel et al., 2008) is integrated into target predictions at http://www.microrna.org where one can search predictions for annotated miRNAs in the main experimental model organisms. These predictions are also integrated with miRBase (Kozomara and Griffiths-Jones, 2014). miRanda analyses miRNA sequence complementarity with 3'UTRs and evaluates binding energy, conservation and binding site position in the 3'UTR. miRanda-miRSVR also identifies non-canonical and non-conserved sites (Betel et al., 2010; Betel et al., 2008).

MIRZA & MIRZA-G (http://www.sib.swiss/zavolan-mihaela/services)

These tools are being developed in Mihaela Zavolan's lab and can be used for the prediction of miRNA targets and siRNA off-targets on a genome-wide scale. MIRZA is biophysical

model of microRNA-target interaction that enables accurate identification of microRNA targets, particularly from Argonaute-CLIP data (Khorshid et al., 2013). MIRZA-G employs both the MIRZA biophysical model as well as other features to predict microRNA target sites genome-wide (Gumienny and Zavolan, 2015). MIRZA-G performed better on a benchmark test than Targetscan Context+ and DIANA-microT-v3 (Gumienny and Zavolan, 2015) making it a good choice for predicting canonical and non-canonical miRNA target sites as well as siRNA off-target sites.

Target prediction for plant miRNAs

Target prediction for plant miRNAs differs from prediction of miRNA targets in animals because of frequent highly complementary targets of plant miRNAs. A pioneering study of miRNA-mediated repression in plants revealed near-perfect complementarity between *Arabidopsis* miRNAs and their targets suggests suggesting that many plant miRNAs act similarly to siRNAs and direct mRNA cleavage (Rhoades et al., 2002). Consequently, miRNA target prediction in plants (for a recent review, see, for example, (Mishra et al., 2015)) is routinely performed as a relatively simple search for highly complementary mRNA sequences without a specialized target prediction algorithm. For example, Singh et al. (Singh et al., 2016) used for miRNA target prediction in ginger (*Zingiber officinale*) the following three simple criteria, which could be written into a simple search script:

1) not more than four mismatches allowed between predicted mRNAs and target gene.
2) no mismatches allowed for 10th and 11th positions of complementary site (a cleavage site).
3) maximum 4 GU pair was allowed in the complimentary alignment.

Some authors even use for searching sequence similarity between a plant miRNA and mRNAs the Basic Local Alignment Search Tool algorithm (Huang et al., 2014a). A specific plant-miRNA-target analysis server is psRNATarget: a plant small RNA target analysis server (Dai and Zhao, 2011), which can be used not only for miRNAs but also for other plant small RNA analysis (Guzman et al., 2013; Huang et al., 2014b; Kumar et al., 2014). Other authors use general target prediction algorithms such as Miranda or RNAhybrid either alone (Shweta and Khan, 2014) or in more complex arrangements (Kurubanjerdjit et al., 2013).

A systematic evaluation of tools to predict targets of miRNAs and siRNAs in plants was provided by Srivastava et al. who compared 11 computational tools in identifying genome-wide targets in *Arabidopsis* and other plants. Among them, Targetfinder was the most efficient in predicting 'true-positive' targets in *Arabidopsis* miRNA-mRNA interactions but performed much worse when analyzing data from non-*Arabidopsis* species. (Srivastava et al., 2014). Furthermore, combination of Targetfinder and psRNATarget provides high true positive coverage, whereas the intersection of psRNATarget and Tapirhybrid outputs deliver highly 'precise' predictions. All evaluated tools yielded a large number of 'false negative' predictions in non-*Arabidopsis* datasets (Srivastava et al., 2014).

Targets of plant miRNAs, that induce sequence-specific RNAi-like cleavage, can be further identified by employing degradome sequencing, a method determining RNA termini.

Thus, in mRNAs cleaved by a miRNA after its 10th nucleotide, one would observe alignment of RNA termini matching the predicted miRNA binding site. This strategy complementing bioinformatics description is further described in the following section.

Common experimental approaches for identification of targets of small RNAs

Bioinformatic target prediction is probabilistic. In other words, bioinformatics predictions identifies a set of putative small RNA targets, which fit certain set of criteria and are assigned a certain probability of being targeted by a specific small RNA. At the same time, each prediction yields positive and false negative results. A common problem in bioinformatic prediction is reliable prediction of non-canonical targets, whose recognition does not involve a complete seed match and, to a lesser extent, prediction of targets recognized through non-conserved binding sites. It is common that researchers aiming at target identification start with bioinformatics prediction and become entangled in the net of prediction tools and generate partially overlapping lists of predicted targets. This strategy is inherently biased towards canonical conserved miRNA binding sites and the highest scoring targets will have more than one such a site. However, this strategy is problematic for identification of the full set of targets.

Accordingly, more reliable identification of small RNA targets usually combines bioinformatics and experimental approaches. (Chen et al., 2015; Tarang and Weston, 2014; Thomson et al., 2011).

High throughput expression analysis

High throughput analysis (expression arrays, RNA sequencing or high-throughput proteomics) can complement target prediction in different ways. One can manipulate the miRNA pathway by various means (reviewed, for example in Svoboda, 2015), such as miRNA overexpression, knock-out or inhibition by complementary oligonucleotides (so-called antagomirs) and then identify correlations between target prediction and their actual behavior (e.g. Krutzfeldt et al., 2005; Lim et al., 2005; Sood et al., 2006). However, these strategies yield only correlative results, i.e. do not directly detect smallRNA:targetRNA interaction.

Small RNA capture strategies

These strategies can be used to identify either mRNAs bound by a small RNA or small RNAs bound to a selected mRNA. Identification of targets of a small RNA employs delivery of a tagged small RNA (e.g. biotinylated miRNA) followed by an affinity capture to co-purify targets (Baigude et al., 2012; Orom and Lund, 2007; Tan and Lieberman, 2016). These strategies are prone to artifacts because delivery of biotinylated small RNAs can create nonphysiological conditions and affinity purification could be influenced by the binding site context.

Identification of small RNAs bound to a selected mRNA (e.g. miR-CATCH or miRIP methods) employs capture of a selected RNA with associated small RNA using a complementary

oligonucleotide, which can be used for affinity capture (e.g. a biotinylated complementary oligonucleotide or a complementary oligonucleotide covalently bound to a magnetic bead) (Su et al., 2015; Vencken et al., 2015). This strategy is suitable for detailed analysis of miRNA-mediated regulation of a specific mRNA but not for a transcriptome-wide target assessment. One variant employs a tested mRNA fused with a common sequence (EGFP) allowing for using the same set of biotinylated DNA anti-sense oligonucleotides for analyzing different mRNAs allowing for increasing the throughput (Wei et al., 2014).

miRNA extension

This strategy employs miRNA extension with a reverse transcriptase on endogenous target mRNAs. Purified hybrid 3'-cDNA-miRNA-5' molecules are used in a second round of reverse transcription and sequenced (Vatolin et al., 2006). However, this method is prone to artifacts stemming from the variability of miRNA:target mRNA base pairing, which would result in highly variable efficiency of reverse transcription priming.

Immunoprecipitation of small RNA:target RNA complexes

There is a large number of immunoprecipitation strategies aimed at purifying small RNA:target RNA complexes, usually by immunoprecipitating them through an Argonaute protein. Initial experiments immunoprecipitated native Argonaute complexes without including a cross-linking step; immunopurified RNAs were analyzed on microarrays (Easow et al., 2007; Hendrickson et al., 2008; Karginov et al., 2007). An adaptation of Argonaute immunoprecipitation for detection of specific miRNA targets is a RIP competition assay wherein anti-miR is titrated into cytosolic extracts prior to Argonaute immunoprecipitation. Direct target transcripts displaced by anti-miR are then identified based on their depletion from IP fraction (Androsavich and Chau, 2014)

The immunoprecipitation strategy was further developed into a number of methods for isolation of small RNAs bound to their targets, which include high throughput sequencing of crosslinking immunoprecipitation (HITS-CLIP) and crosslinking ligation and sequencing of hybrids (CLASH) methods (reviewed more detail in Broughton and Pasquinelli, 2016; Jaskiewicz et al., 2012).

There are several modifications of the basic HITS-CLIP. An improvement of the basic HITS-CLIP approach (Chi et al., 2009; Zisoulis et al., 2010) came with crosslinking based on photoactivatable nucleosides such as 4-thiouridine, a CLIP modification known as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) (Hafner et al., 2010; Hafner et al., 2012). PAR-CLIP offers more efficient crosslinking, hence up to three orders of magnitude better RNA recovery than HITS-CLIP (Hafner et al., 2010). Furthermore, PAR-CLIP also allows for precise localization of miRNA binding site as cross-linked 4-thiouridine marks the cross-linked site with frequent thymidine to cytidine change, which is revealed by deep sequencing (Hafner et al., 2010).

Another modified strategy is covalent ligation of endogenous Argonaute-bound RNAs crosslinking immunoprecipitation (CLEAR-CLIP) which enriches miRNAs ligated to their endogenous mRNA targets (Moore et al., 2015). CLEAR-CLIP approach is in principle

the same as the above-mentioned CLASH (Helwak et al., 2013; Helwak and Tollervey, 2014). Adding ligation of miRNAs to their mRNA targets yields chimeric reads allowing for robust detection miRNA:target RNA interactions occurring *in vivo*.

Data mining of CLIP data provides not only a comprehensive list of miRNA:target mRNA interactions but also provides insights into the principles governing these interactions, which in turn facilitate further improvement of target prediction algorithms. For example, an *in vivo C. elegans* data set and reanalysis of published mammalian AGO-CLIP data yielded approximately 17,000 miRNA:target site interactions. This strategy identified canonical, noncanonical, and nonconserved miRNA:targets with about 80% of miRNA interactions having perfect or partial seed complementarity (Grosswendt et al., 2014). Another comprehensive analysis of 34 Argonaute HITS-CLIP datasets from human and mouse cells revealed that many heteroduplexes are "non-canonical" i.e. their seed region comprises G:U and bulge combinations (Clark et al., 2014).

CLIP strategies are nowadays popular for high-throughput analysis of physiological miRNA targets (Chi et al., 2012; Chi et al., 2009; Clark et al., 2014; Grosswendt et al., 2014; Haecker and Renne, 2014; Hafner et al., 2010; Imig et al., 2015; Leung et al., 2011; Liu et al., 2014a; Marin et al., 2012; Zisoulis et al., 2010) and it is accompanied with a number of algorithms and databases facilitating identification of miRNA targets in high-throughput CLIP data (Balaga et al., 2012; Bandyopadhyay et al., 2015; Chou et al., 2013; Erhard et al., 2013; Guo et al., 2015; Hsieh and Wang, 2011; Hsu et al., 2015; Liu et al., 2013; Paraskevopoulou et al., 2013a; Rennie et al., 2014; Wang et al., 2013; Wang et al., 2014; Xie et al., 2014; Yang et al., 2011).

Degradome analysis

It was mentioned above in the section discussing target prediction for plant miRNAs that when small RNA-target RNA interaction results in RNAi-like cleavage, identification of targets can be experimentally augmented by degradome sequencing. This sequencing allows for identification of RNA termini, including those created by RNAi-like cleavage. Thus, if there are mRNAs cleaved by a miRNA after its 10th nucleotide, one would observe alignment of RNA termini matching the predicted miRNA binding site. Degradome analysis usually defines a category of transcripts predicted to be endonucleolytically cleaved and then are cleavage positions compared to predicted miRNA binding sites (Ding et al., 2016; Fan et al., 2016; Li and Sunkar, 2013; Shao et al., 2013; Wang et al., 2016; Xing et al., 2014). Degradome analysis and target prediction has been integrated in to a web resource comPARE for plant miRNA target analysis (Kakrana et al., 2014). Degradome analysis can be also used in animals to identify rare miRNA targets suppressed by slicing (Park et al., 2013).

Conclusions

Given the nature of the interaction between small RNAs and their target RNAs, target prediction will always yield putative targets with partial complementary. For seed-mediated interactions, there can easily be hundreds of targets predicted for any small RNA acting

as a miRNA. This is due to the combination of the following facts: a) a specific hexamer sequence occurs in a random sequence with a theoretical frequency of 1/4096 and b) exons of protein-coding genes constitute 70–80 megabases of well-annotated mammalian genomes (and exome size of eukaryotic genomes might not be dramatically smaller than that). Therefore, a hexamer would occur in a mammalian exonic sequence on average ~20 000x and if 1% of these hexamers would fit other target site prediction criteria, that would leave on average 200 potential binding sites.

The bottom line is that applying a minimal base pairing criterion for miRNA-like interaction will identify a number of potential targets in any eukaryotic organism. At the same time, sequence based target prediction is insufficient to assess whether there will be target repression induced by a specific small RNA when introduced into an animal or mammal because there is a number of other critical parameters, which must be considered. Two of them stand out above anything else: 1) the amount of the specific small RNA loaded on Argonaute proteins, and 2) target site accessibility. Thus, target assessment of small RNAs needs to address these two parameters. While target accessibility can be considered a relatively common feature for all organisms since the same rules would apply for RNA folding and interference caused by RNA binding proteins (translation machinery etc.), loading of a small RNA onto Argonaute proteins depends on factors which may dramatically differ between different organisms. For example, organisms that exhibit environmental RNAi would be much more prone to the uptake of small RNAs. Factors such as length, chemical modifications, or terminal nucleotides of a small RNA, could underlie differences in sorting and loading to the various Argonaute proteins. Thus, possible fates of a specific small RNA in a specific organism are difficult to predict and should be tested experimentally. The most informative parameter is the amount (number of molecules) of a small RNA in question, which would be loaded on an Argonaute protein (e.g. AGO1 and AGO2 in animals), because it could be compared with known kinetic data to assess the strength of potential repressive effects it could achieve *in vivo*.

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